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6. AUTHOR(S)
Dr. Thomas C. Bruice

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, Santa Barbara
Department of Chemistry
Santa Barbara, CA 93106

8. PERFORMING ORGANIZATION REPORT NUMBER

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Solid Phase Synthesis of DNG has been perfected. Also, solid phase synthetic procedures for mixed DNA and DNG sequences are now at hand. Studies of the protection of the DNA component, form exo- and endo-nuclease hydrolysis, DNG moiety have been initiated.
Replacement of the negative phosphodiester linkages of DNA by positive S-Methyl thiourea linkers provides another putative phylogenetic class (DNmt) of mRNA binding molecules.

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FINAL REPORT

GRANT #: ONR N00014-96-1-0123

PRINCIPAL INVESTIGATOR: Thomas C. Bruice

INSTITUTION: University of California at Santa Barbara

GRANT TITLE: DNG and RNG Phylogenetic Single Cell Probes

AWARD PERIOD: 1 Nov. 1995 to 30 Sept. 1998

OBJECTIVE: The subject of this grant is the synthesis and study of DNA and RNA mimics in which the negatively charged phosphate diester linkages of DNA and RNA [-O-(PO_2^-)-0-] are replaced by positively charged linkers as the guanido linker [-NH-(C=NH_2^+)-NH-] in DNG and RNG.[1,2]

APPROACH: The first DNG and RNG studies were carried out with the financial assistance from ONR N00014-90-J-4132. By the time the present grant commenced in 1995 we had shown that short sequences of DNG and RNG with nucleobase T could be synthesized by stepwise procedures in solution [$(Tg)_4T$]. We had also shown that $(Tg)_4T$ formed segments of high melting triple helix ($[(Tg)_4T]_2 \cdot [(Ap)_4A]$) on reaction with poly-A but did not react with poly-C, poly-G or poly-I -- as judged by increase in absorbance. Thus, at the onset we had some reasons to believe there existed a degree of fidelity in base recognition as a prerequisite to helix formation.

Our stated goal, for the research we now report on, has been to master the technology required to synthesize requisite DNG and/or RNG oligos complementary to RNA signatures of salt water bacterial rRNA. These DNG and RNG oligomers would be offered as tools for the phylogenetic classification and detection of salt water bacteria. To this end Professor Edward DeLong[3,4] was to join us with studies of *in situ* hybridization in single cells. There is a great deal of work to do here because we must not only perfect a number of synthetic procedures, including solid phase synthesis of needed DNG/RNG oligos, but we must determine the thermodynamics and kinetics of formation and the physical

characteristics of DNG and RNG double and triple strand helices with complementary RNA oligos. We must also determine the fidelity of recognition by DNG and RNG of base sequences in complementary RNA. One patent has been obtained and a second patent has been filled for.

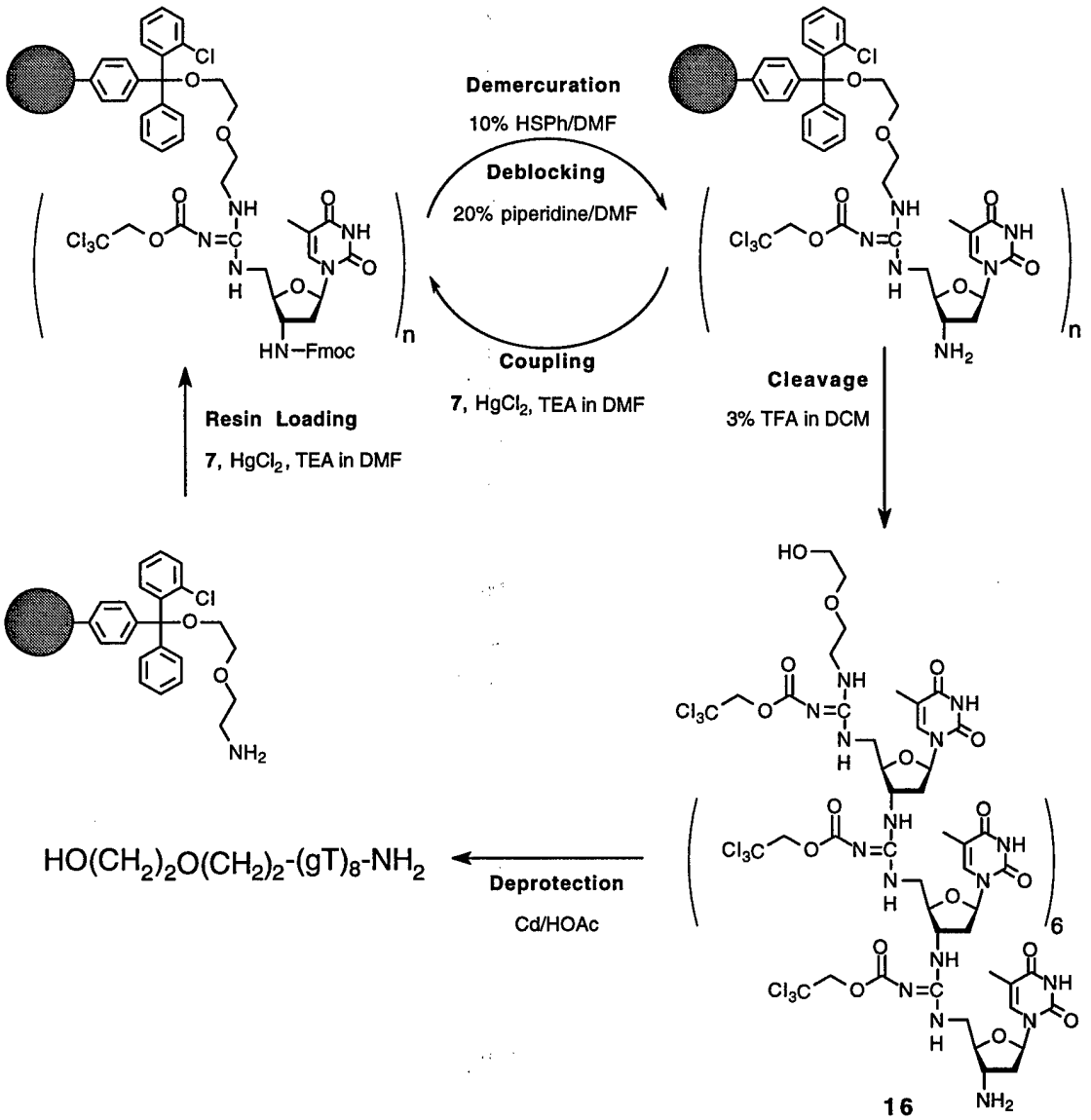
ACCOMPLISHMENTS & CONCLUSIONS:

To date we have concentrated on the use of the nucleobase thymine in the development of solid phase synthesis of DNG and RNG. There would simply be too many variables if extension experiments were carried out simultaneously using the nucleobases T, A, G, C, and U. The step by step synthesis in solution of DNG and RNG sequences[1,2,5] is laborious and the yields are such that chain lengths greater than five are not practical objectives. A great deal of effort has paid off in the development of a solid support synthesis capable of the addition of one base every four hours. Yields of T oligomers [$(Tg)_nT$] of n = 12 are obtained in 50% purity (easily purified by HPLC).[6] The synthetic procedure is shown in Chart I.[7] The nucleotide coupling step in the synthesis involves the attack of a terminal 3'-amine upon an electronically activated 5'-carbodiimide to create a protected guanidinium internucleotide linkage. The activated carbodiimide is synthesized in-situ by the addition of $HgCl_2$ to a solution of a nucleotide which possesses a 3'-amine protected with an Fmoc group and a N,N' unsymetrically substituted thiourea in the 5'-position with the nucleotide attached to the other. The Mg^{2+} abstracts the sulfur from the thiourea creating the carbodiimide. The HgS precipitate is removed by washing with a solution of thiophenol.

Solid phase procedures have been worked out whereby guanidinium linkers (-NH-C(=NH_2^+)-NH-) can be included into olgonucleotides along with phosphodiester linkages (-O-(PO_2^-)-O-)[8]. This is accomplished by the synthesis of 3'-HOT-g-TOH-5', 3'-HOA-g-AOH-5' etc which can be incorporated as subunits in the synthesis of DNA. For this purpose, standard phosphoramidite chemistry and automated solid phase synthesis was employed to prepare the 18mers:

9. 5'-d(**T*TGTTAGTT*TTCTTGT*TT**)-3'
10. 5'-d(**T*TGTTAGTTTTCTTGT*TT**)-3'
11. 5'-d(TTGTTAGT**T*TTCTTGTTT**)-3'

Chart I


Demercuration
10% HSPh/DMF
Deblocking
20% piperidine/DMF
Coupling
7, HgCl2, TEA in DMF
Resin Loading
7, HgCl2, TEA in DMF
Cleavage
3% TFA in DCM
Deprotection
Cd/HOAc
HO(CH2)2O(CH2)2-(gT)8-NH2
16

Capping at the terminal 5'- and 3'-ends, as with 9 & 10, provides resistance to exonuclease hydrolysis. At low ionic-strength 9 exhibits tighter binding to complementary DNA than does DNA of same sequence as 9. ODN 11 undergoes only partial hydrolysis by exonucleases. This partial hydrolysis of 11, having guanidinium at the center indicates that phosphodiester linkages around guanidinium are stable to exonuclease cleavage.

A detailed kinetic and thermodynamic study of the association of short strand DNA oligomers, composed of A and G nucleobases {$A_5G_3A_5$ $GA_4G_3A_4G$, $G_2A_3G_3A_3G_2$, and $G_2A_2G_5A_2G_2$}, with the DNG $d(Tg)_4$-T-azido has been carried out.[9,10] There is a better stabilization of the triplexes {$(d(Tg)_4$-T-azido$)_2 \cdot (G_nA_mG_oA_mG_n)$} at low ionic strength and low percent G. The variation in stability falls over a range of -8 to -12 kcal/mol with large negative values at low ionic strength. The standard molar enthalpies $\Delta H°(288) = Eon - E_{off}$ are between -48.5 and -22.7 kcal/(mol base). Compensation relates the values of $\Delta H°$ and $\Delta S°$. Computational analysis of the structures of DNG with RNA[11] and DNA[12] in water with periodic boundaries have been carried out to the nanosecond range by molecular dynamic simulations using the EWALD summation method

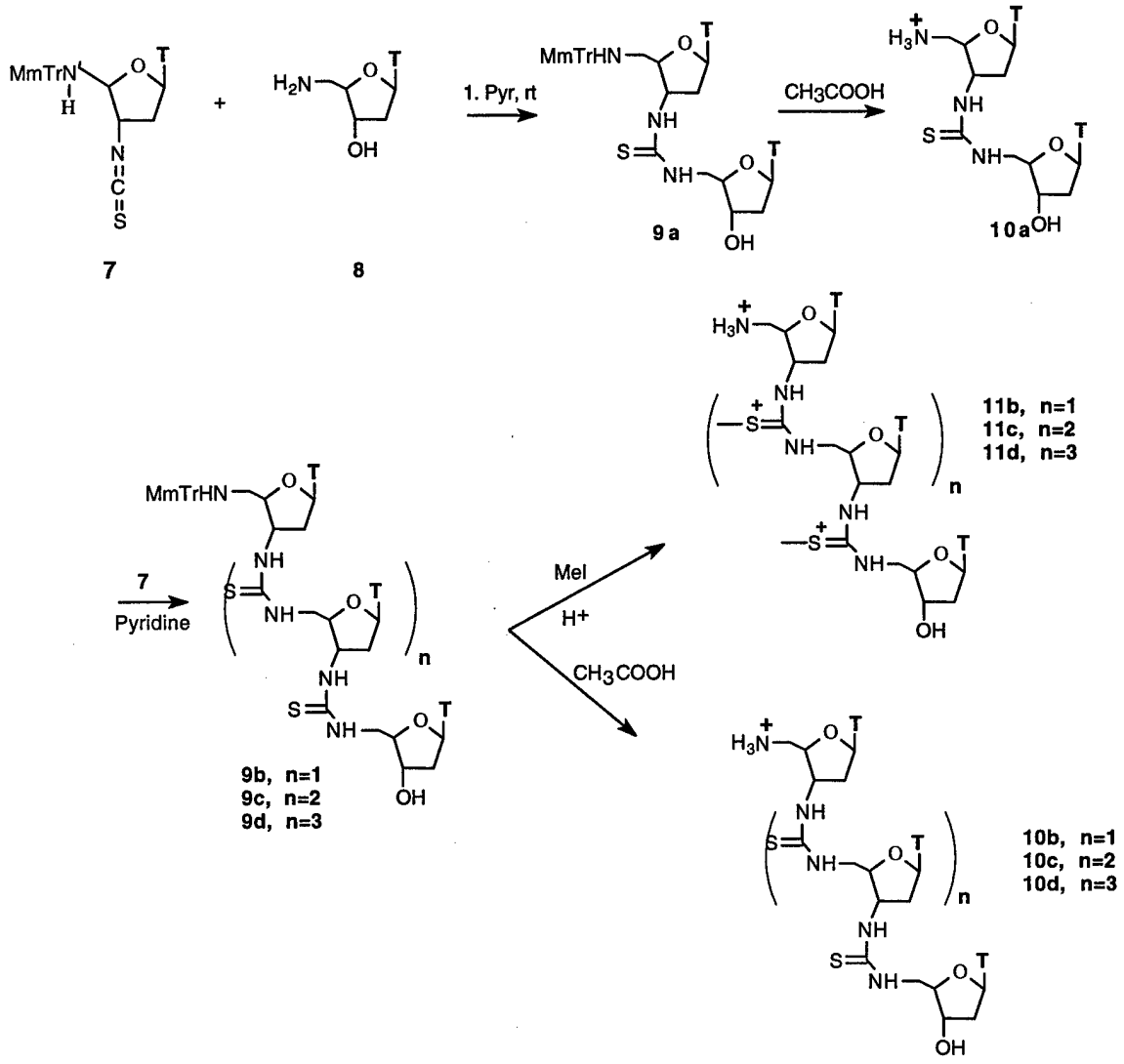
Replacement of the negative phosphodiester linkages of DNA by positive S-Methyl thiourea linkers provides another putative phylogenetic class (DNmt) of mRNA binding molecules (Chart 2).[14] DNmt poly cations have, apparently, the same fidelity in binding of complementary sequences as found with DNG.

SIGNIFICANCE: We have invented two new classes of RNA/DNA binding molecules which are polycations and as such bind more strongly to the poly anionic RNA and DNA oligos. To this time it would appear that fidelity of complementary binding is not jeopardized.

PATENT INFORMATION:

Thomas C. Bruice, R. O. Dempcy & O. Almmarson. POLYNUCLEOSIDE CHAIN HAVING MULTIPLE NUCLEOSIDES, THE NUCLEOSIDES COUPLED BY GUANIDYL LINKAGES, U. S. Patent Number 5,696,253 on December 9, 1997.

Chart 2. Synthetic Plan for T5-mt (**11d**)



PUBLICATIONS:

1. R. O. Dempcy, K. A. Browne and T. C. Bruice, Synthesis of the Polycation Thymidyl DNG, It's Fidelity in Binding Polyanionic DNA/RNA, and the Stability and Nature of the Hybrid Complexes, . Am. Chem. Soc., 117, 6140 (1995).
2. K. A. Browne, R. O. Dempcy, T. C. Bruice, Binding Studies of Cationic DNG to RNA Homopolynucleotides, Proc. Natl. Acad. Sci (USA), `92, 7051 (1995).
3. E. F. DeLong, G. S. Wickham, N. R. Pace, *Science* 243, 1360 (1989).
4. a. E. F. DeLong, G. S. Wickham, N. R. Pace, *Science*, 243, 1360 (**1989**).
   b. E. F. De Long, *NATO ASI Series*, G27, 237 (**1991**). c. D. L. Distel, E. F. DeLong, J. B. Waterbury, *Appl. Environ. Microbiol.* 57, 2376 (**1991**).
   d. E. L. Lim, L. A. Amaral, D. A. Carbon, E. F. DeLong, *Appl. Environ. Microbiol.* 59, 1647 (**1993**).
   e. E. F. DeLong, K. Y. Wu, B. B. Prezelin, R. V. M. Jovine, *Nature*, 371 695 (**1994**).
5. R.O. Dempcy, Jia Luo and T.C. Bruice, Design and Synthesis of Ribonucleic Guanidine: A polycationic analog of RNA, *Proc. Natl. Acad. Sci* (USA), 93, 4326 (1996).
6. B. Linkletter & T. C. Bruice, Solid Phase Synthesis of Oligomeric Deoxynucleic Guanidine (DNG): A Polycationic Analogue of DNA, *Bioorganic & Med. Lett.*. 8, 1285 (1998).
7. B. Linkletter, I. Szabo & T. C. Bruice, ImprovedSolid Phase Synthesis of Deoxynucleic Guanidine (DNG) Oligamers nsandf Melting Point and Circular Dichroism Analysis of Binding Qfidelity of Octameric Thymidyl Oligamers with DNA Oligamers, *J. Am. Chem. Soc. Submitted.*. 8, 1285 (1998).
8. D. A. Barawkar & T. C. Bruice, Synthesis, Biological Properties and nuclease resistance properies of mixed backbone oligodeoxynucleotides containing cationic internucleoside guanidinium linkages: DNA/DNG chimeras, *Proc. Natl. Aca. Sci* (USA) 95, 11047 (1998).
9. A. Blasko, R. O. Dempcy, E. E. Minyat & T. C. Bruice, Association of Short Strand DNA Oligomers with Guanidinium Linked Nucleosides (DNG). A Kinetic and Thermodynamic Study, J. Am. Chem. Soc., 118, 7892 (1996). .
10. A. Blasko, E. E. Minyat, R. O. Dempcy & T. C. Bruice, Fidelity of Binding of the Guanidinium Nucleic Acid (DNG) d$(Tg)_4$-T-azido with Short Strand DNA Oligamers {$A_5G_3A_5$, $GA_4G_3A_4G$, $G_2A_3G_3A_3G_2$,$G_2A_2G_5A_2G_2$} with a Guanidinium Linked (DNG) Nucleoside. A Kinetic and Thermodynamic Study, Biochemistry 36, 7821 (1997).
11. J. Luo & T. C. Bruice, Nanosecond Molecular Dynamics Study of a Polycation Ribonucleic Guanidine (RNG) Duples with a Complimentary DNA Oligomer Strand, J. Am. Chem. Soc., 119, 6693 (1997).
12. J. Luo & T. C. Bruice, Nanosecond Molecular Dynamics of Hybrid Triplex and Duplex of Polycation Deoxyribonucleic Guanidine (DNG) Strands with a Complimentary DNA Strand, *J. Am. Chem. Soc.*, 120, 1115 (1998).
13. R. A. Torres, O. Almarsson and T. C. Bruice, Molecular MechanicsCalculations of the Riboacetal Internucleotide Linkage in Double and Triple Helices, Proc. Natl. Acad. Sci (USA), 93, 6875 (1996).
14. D. P. Arya & T. C. Bruice, Replacement of the Negative Phosphodiester Linkages of DNA by Positive S-Methyl Thiourea Linkers: A Novel Approach to Putative Antisense Agents, *J. Am. Chem. Soc.*, 120, 6619 (1998).